Influence of Various PGRs on Physiological Attributes, Yield Characteristics and Essential Oil Constituents of *Mentha piperita* L.

DISSERTATION

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BY

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Dedicated

To

My Loving Parents

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Certificate

This is to certify that the dissertation entitled **"Influence of Various PGRs on Physiological Attributes, Yield Characteristics and Essential Oil Constituents of *Mentha piperita* L."** submitted in partial fulfilment of the requirements for the degree of **Master of Philosophy** in Botany is a faithful record of the bonafide research work carried out at the Aligarh Muslim University, Aligarh by **Ms. Daraksha Khanam** under my guidance and supervision and that no part of it has been submitted for any other degree or diploma.

(Firoz Mohammad)
Supervisor of Research

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(DARAKSHA KHANAM)

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

Medicinal and aromatic plants (MAPs) occur in innumerable forms and play an important role in the socio-cultural, spiritual and health care necessities of people. India is considered as a treasure house of MAP species and probably has the oldest, richest and most diverse cultural traditions in the use of medicinal plants. About 7500, the half of the country's 17000 endemic plant species, are used in ethnomedics (Shankar and Majumdar, 1997). Medicinal plants have been used for centuries as remedies for human diseases because they contain chemical components of therapeutic values (Nostro, 2000). MAP species are still largely gathered and collected from the wild and relatively a few are cultivated in farmland. The exploitation from the nature coupled with increasing urbanization have led to steady erosion and loss of MAP biodiversity from the natural habitat. It is, therefore, necessary that these valuable plant species should not only be preserved but their cultivation practices should also be developed in order to meet the entire demand of the domestic industries and also to harness the bright prospect for current export. Essential oils and extracts obtained from many plants have recently gained a great popularity and scientific interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multi-purpose functional use (Ormancey, 2001). These are valuable natural products used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, spices, nutrition and development of medicines against various diseases and also for the development of industrial products (Newall, 1996; Buchbauer, 2000).

Mentha piperita L. (peppermint) is one of the important MAPs belonging to the family Lamiaceae and it is best known for its essential oil. Lamiaceae contains about 200 genera, 2000 species of herbs and 5000 species of low shrubs (Hedge, 1992). Peppermint is one of the world's oldest medicinal herbs and is used in both Eastern and Western traditions. Ancient Greek, Roman, and Egyptian cultures used this herb in cooking and medicine. Peppermint leaves and oil are used for folk medicine, as flavouring agent, and in cosmetics and pharmaceutical products throughout the world (Foster, 1996). Peppermint oil is the most extensively used of all the volatile oils (Murray, 1995).Taking into account the commercial importance of the plant as a rich source of essential oil in the modern system of medicines, it seems highly desirable to

make efforts to raise the cultivation of plant and to amplify the production of its oil on scientific lines. In this regard various plant growth regulators (PGRs) can be used to enhance its production (El-Keltawi and Croteau, 1986, 1987; Zheliazkov, 2010; Kumar *et al.*, 2012; Abdou and Mohamed, 2014).

PGRs are chemicals that control plant growth and development. They stimulate growth and terpenoid biosynthesis in various aromatic plants, which results in beneficial changes in both quality and quantity of terpenoids (Zlatev *et al.*, 1978, 1990; El-keltawi *et al.*, 1987; Sangwan *et al.*, 2001; Prins *et al.*, 2010; Zheljazkov *etal.*, 2010; Sharafzadeh and Zare, 2011). They influence the interrelationship between primary and secondary metabolism leading to increased biosynthesis of secondary products. PGRs are known to improve the source-sink relationship, translocation of photo-assimilates and thereby photosynthetic ability of the plants and thus play a significant role in realization of high productivity levels of crop plants (Roitsch and Ehneb, 2000; Iqbal *et al.*, 2011).

Gibberellins like gibberellic acid (GA_3), auxins namely indole acetic acid (IAA) and indole butyric acid (IBA), cytokinins such as kinetin (Kn) and benzyl amino purine (BAP), triacontanol (Tria), methyl jasmonate (MJ), salicylic acid (SA) and cycocel (CCC) are important PGRs as far as the realization of plants is concerned. Gibberellins are important for their role in seed germination, bolting and induction of flowering while auxins have various roles in cell elongation, cell division, dormancy and apical dominance, whereas cytokinins play crucial role in the cell cycle by controlling G1/S transition and increasing the number of replication origins during S phase to control G2/M transition (Kim and Park, 2007). Cytokinins are also indispensable for seed germination, cotyledon expansion, shoot branching, vascular differentiation, senescence delay, breaking of dormancy, promotion of pigments and chloroplast development (Taiz and Zeiger, 2010). Tria is involved in regulation of photosynthesis, water and mineral nutrient uptake (Chen *et al.*, 2003).MJ have various roles in the induction of biosynthesis of secondary metabolites (Choi *et al.*, 2005).SA plays a role in growth and development, photosynthesis, transpiration and seed germination (Hayat *et al.*, 2007). It functions as an endogenous signal mediating compound which render local and systemic plant defence responses against pathogens (Vicente and Plasencia, 2011). CCC acts as growth retardant and increase tolerance against various stresses (Sinha, 2013).

Because of their various important roles in growth and development of plants, PGRs are used for improvement of crop yield potential and quality of production and are described as the most important tools for the agriculturist to increase crop yield.

Keeping the importance of essential oil in view, it was decided to cultivate peppermint on scientific lines under local conditions of Aligarh in western Uttar Pradesh, India. In this regard a pot experiment was conducted in a net-house of the Department of Botany, AMU, Aligarh (U.P.) with the following aim:

- To study the effect of foliar application of various PGRs on growth, physio-biochemical, yield and quality characters of peppermint.

The details of the pot experiment planned with the above objective are given in Chapter 3 (pp. 19-32).

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

This chapter deals with the general description of peppermint and the selected PGRs as also the effect of different PGRs on the performance of *Mentha* and other essential oil producing plants.

2.1 Peppermint

Peppermint is an important medicinal aromatic perennial herb belonging to the family Lamiaceae. It is the oldest known medicinal plant species, apart from its potential use as a flavouring agent. The plant is aromatic, stimulant, stomachic, carminative and used for allaying nausea, flatulence, headache and vomiting. The active constituents of peppermint leaves are about 50% essential oil (mainly menthol), 19% total poly-phenolic compounds and 7% total hydroxyl-cinnamic (Duband *et al.*, 1992; Maffei *et al.*, 1999). The remarkable aromatic and remedial properties of its oil render a valuable source of foreign exchange.

2.1.1 Botanical description

Peppermint is a sterile, perennial herb originating from a hybridisation between watermint (*Mentha aquatica*) and spearmint (*Mentha spicata*). It is an herbaceous rhizomatous perennial plant growing to 30-90 cm tall, with smooth stems. The rhizomes are wide-spreading, fleshy and bear fibrous roots. It has stalked smooth dark green leaves. The leaves are 4-9 cm long and 1.5-4 cm broad, dark green with reddish veins, and with an acute apex and coarsely toothed margins. The leaves and stems are usually slightly fuzzy. The entire plant has a very characteristic sharp, mint odour, because of the presence of the volatile oil. The flowers are purple, 6-8 mm long with a four-lobed corolla (about 5 mm diameter) and are produced in whorls (verticillasters) around the stem, forming thick, blunt spikes. Flowering is from mid to late summer.

2.1.2 Origin and distribution

Peppermint plant is thought to be of Mediterranean origin. Dried leaves were found in the Egyptian pyramids dating back to 1000 BC. It is mentioned in the Icelandic pharmacopoeias of the thirteenth century, but only came into general use in the

medicine of western Europe about the middle of the eighteenth century. Peppermint is much cultivated in many countries of Europe and western and central Asia for the production of menthol. Northern Africa is also a main cultivation area. India became the world's leading producer and exporter of mint oil after green revolution and contributes about 80 per cent of total world production. At present the crop is mainly cultivated in Tarai regions, Indogangetic plains, Punjab and North-western India.

2.1.3 Systematic position

According to the system of classification of Cronquist (1981), peppermint occupies the following systematic position:

Kingdom Plantae
Division Magnoliophyta
Class Magnoliopsida
Subclass Asteridae
Order Lamiales
Family Lamiaceae
Genus *Mentha*
Species *piperita*

2.1.4 Cultivation

Peppermint is grown in cool to temperate regions. Long day lengths with warm to hot conditions and cool nights are required for the right balance of oil compounds to be produced during the growing phase. It needs adequate rainfall on a regular basis in excess of 1000 mm. Frequent and adequate irrigation is required, which is used to supplement rainfall. It generally grows best in moist, shaded locations and expands by underground stolons. The best soils are deep, well-drained and rich in humus with good moisture retention. The soil pH is between 5.5 and 7.0. Timing of harvest is critical to the quality of the oil. Optimum oil yield and quality are usually obtained when 10% of the crop is in the flowering stage. The leaves and flowering tops are used and they are collected as soon as the flowers begin to open. Harvesting is generally carried out on a dry sunny day in the late morning when all traces of dews have disappeared.

2.1.5 Main constituents of peppermint oil

Over 200 different constituents have been identified in peppermint oil (Lawrence and Shu, 1989; Chialva *et al.*, 1993; Court *et al.*, 1993). The essential oil of peppermint (up to 0.3% to 1% in the dried leaves) is mostly made up from menthol (55.8%), menthone (20%) and menthyl esters (9.1%) and also monoterpene derivatives pulegone, piperitone, menthofuran (Handa *et al.*, 1964; Anonymous, 2004). Menthol (Fig. 1) and menthyl acetate (Fig. 3) are responsible for the pungent and refreshing odour. They are mostly found in older leaves and are preferentially formed during long daily sunlight periods. However, the ketones menthone (Fig. 2) and pulegone (and menthofuran) have a less delightful fragrance. The ketones appear to higher fraction in young leaves and their formation is preferred during short days.

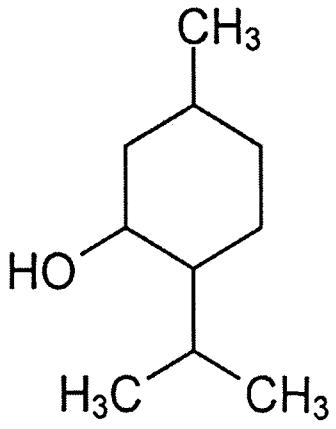

Fig. 1. Menthol

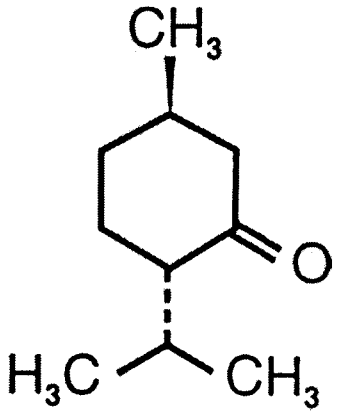

Fig. 2. Menthone

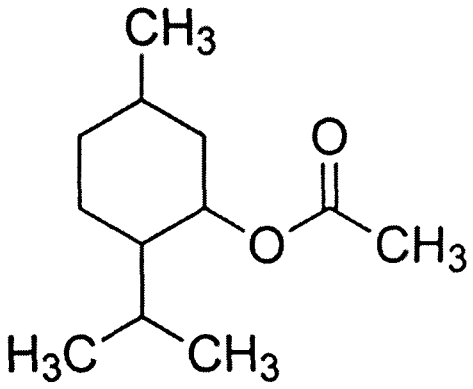

Fig. 3. Menthyl acetate

2.1.6 Medicinal and commercial uses

Main medicinal properties of peppermint are antiseptic, antispasmodic, carminative, cephalic, stimulative and stomachic. Peppermint and its essential oil are effective in the treatment of nervous disorder and mental fatigue. Peppermint is used to disguise the taste of unpalatable drugs, as it imparts its aromatic characteristics to the drugs. It may be administered with sugar or in the form of tablet and lozenges. The oil has mild antiseptic and local anesthetic properties. It has anticancerous activity (Kumar *et al.*, 2004). It is used as an external application in rheumatism, neuralgia, congestion, headache and toothache. Peppermint oil is also a soothing remedy for colds and flu. It is used to relieve indigestion, colic, nausea, diarrhoea, Crohns disease, stomach ulcers and ulcerative colitis. (Anonymous, 2004). In aromatherapy, the essential oil is used to stimulate hot and cold nerve endings and increase blood flow. Peppermint oil is most popular for flavoring candy, mouthwashes, toothpaste and lip balm. Leaves of peppermint are used for flavouring tea. Peppermint oil is also used in cosmetics such as bath oils, hair conditioners, lipstick, makeup base, shaving cream, soaps and nail creams.

2.2 Plant growth regulators

PGRs play a crucial role in regulation of plant growth and secondary metabolite production in plants. Auxins, gibberellins, cytokinins, SA and many other classes of compounds have been reported to possess plant growth regulatory activities.

2.2.1 Auxins

2.2.1.1 Indole-3-acetic acid

IAA is the most abundant and the basic auxin. It was isolated from *Avena* coleoptile by Went in 1928. It has been the subject of extensive studies by plant physiologists. In appearance, IAA is a colourless solid. Chemically, IAA is a carboxylic acid in which the carboxyl group is attached through a methylene group to the C-3 position of an indole ring (Fig. 5).

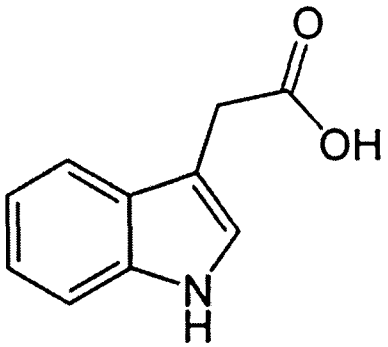

Fig. 5. Indole-3-acetic acid

IAA is produced in actively growing shoot tips, root tips and developing fruit. It causes cell elongation in plants, initiates and promotes cell division, enhances apical dominance, stimulates the formation of new adventitious roots (Sinha, 2013).

2.2.1.2 Indole-3-butyric acid

IBA is a synthetic plant hormone of auxin family and is an ingredient of many commercial horticultural plant rooting products. It is a white to light-yellow crystalline solid. It melts at 125 °C at atmospheric pressure and decomposes before boiling.

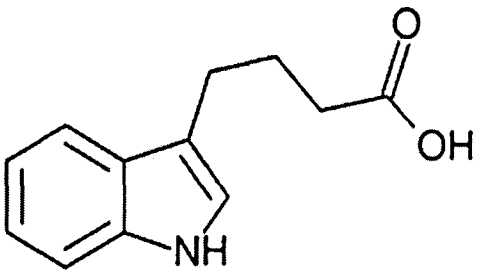

Fig. 6. Indole-3-butyric acid

When applied exogenously, IBA has a variety of different effects on plant growth and development, but the compound is still mainly used for the induction of adventitious roots (Muller, 2000).

2.2.2 Gibberellins

Gibberellins are defined as compounds that contain gibbane skeleton which consists of carbon skeleton with four rings I, II, III, and IV. Hence they are designated as tetra carbocyclic compounds. Over 136 naturally occurring gibberellins have been isolated from fungi, plant and bacteria (Mac Millan, 2002). These are abbreviated as GA with a subscript such as GA_1, GA_2, and GA_3 and so on. They are numbered in the order of their discovery. Of these, GA_3 is commonly available and most widely used in plant physiological research (Noggle and Fritz, 1986). It was first isolated by Yabuta and Sumiki (1938) in Japan, from fungal strains of *Gibberella fujikuroi.*

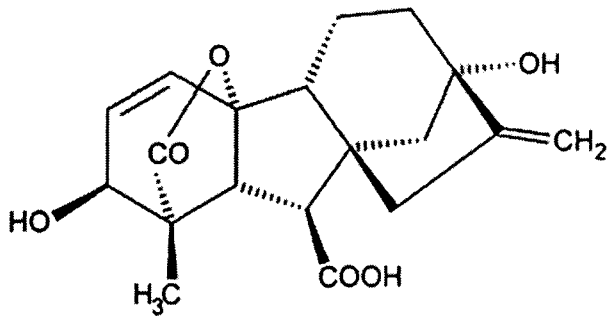

Fig. 4. Gibberellic acid

Gibberellins are widely used phytohormones in horticulture and food industries. They enhances longitudinal growth of internodes of genetically dwarfed plants. Gibberellins are associated with various growth and development processes such as seed germination, stem and hypocotyls elongation, leaf expansion, floral organ development, reduction in time to flowering, increase in flower number and size and induction of some hydrolytic enzymes in the aleurone of cereal grains (Akazawa *et al.*, 1991; Matsuoka, 2003; Swain and Singh, 2005; Khassawneh *et al.*, 2006; Srivastava and Srivastava, 2007).

2.2.3 Cytokinins

2.2.3.1 Benzylaminopurine

BAP is a first-generation synthetic cytokinin that elicits plant growth and development responses, setting blossoms and stimulating fruit richness by stimulating cell division.

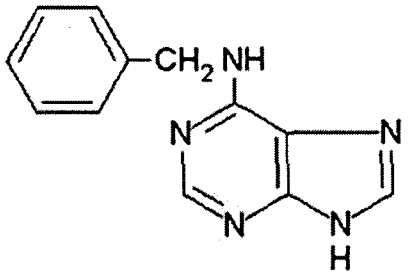

Fig. 7. Benzyl amino purine

It is an inhibitor of respiratory kinase in plants and increases post-harvest life of green vegetables.

2.2.3.2 Kinetin

Kn is a kind of cytokinin. It was originally isolated by Miller and Skoog (1955) as a compound from autoclaved herring sperm DNA. It was given the name kinetin because of its ability to induce cell division. Kn is formed in root tips and are transported upwards via xylem cells (Taiz and Zeiger, 2010).

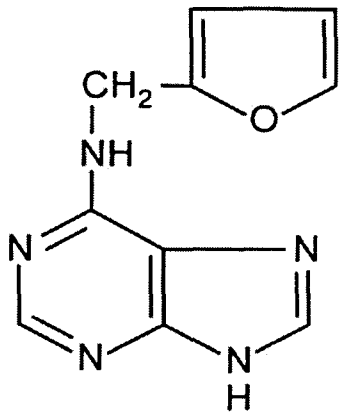

Fig. 8. Kinetin

Besides cell division, it induces synthesis of secondary metabolites, regulates the absorption of nutrients and stimulates the synthesis of RNA and helps in delaying senescence by maintaining protein synthesizing machinery (Sinha, 2013).

2.2.4 Triacontanol

Tria is a saturated long chain alcohol that is known to have growth promoting activities on a number of plants when applied exogenously. It was first discovered by Chibnall *et al.* (1933) as a natural component of epicuticular waxes of *Medicago sativa.*

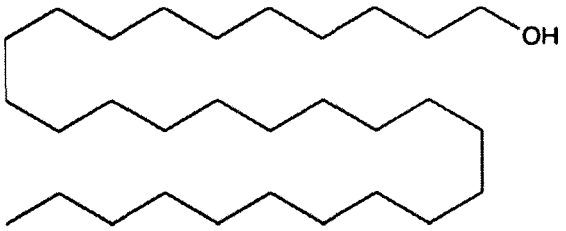

Fig. 9. Triacontanol

Tria has been reported to enhance photosynthesis (Eriksen *et al.*, 1981) and water and mineral nutrients uptake (Ries, 1983, 1985; Ivanov and Angelov, 1997; Chen *et al.*, 2003), regulates activities of various enzymes (Naeem *et al.*, 2011), increases the level of various organic compounds in leaf tissues and involves in the up-regulation of many genes involved in the photosynthetic process (Kumaravelu *et al.*, 2000; Chen *et al.*, 2002, 2003).

2.2.5 Methyl jasmonate

MJ was discovered in 1962 as a sweet-smelling compound from jasmine oil derived from *Jasminium grandiflorum* (Demole *et al.*, 1962).

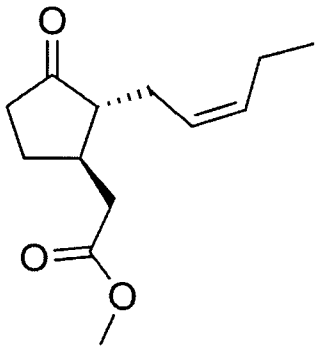

Fig. 10. Methyl jasmonate

It is a volatile organic compound used in plant defense and many diverse developmental pathways such as seed germination, root growth, flowering, fruit ripening and senescence. It induces ethylene-forming enzyme activity, which increases the amount of ethylene to the amount necessary for fruit maturation. In addition, it induces or increases the biosynthesis of many secondary metabolites that play important roles in plant adaptation to a particular environment (Choi *et al.*, 2005).

2.2.6 Salicylic acid

It is known as 2-hydroxy benzoic acid and is one of the diverse groups of phenolic compound. In the year 1828, Buchner isolated salicyl alcohol glucoside (salicin) from willow bark that was later named as salicylic acid by Piria in the year 1838 (Raskin, 1992).

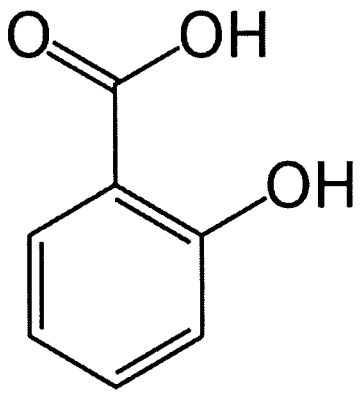

Fig. 11. Salicylic acid

It plays exclusive role in plant growth, thermogenesis, flower induction and uptake of ions. It affects ethylene biosynthesis, stomatal movement. It also enhances the level of photosynthetic pigment, photosynthetic rate and modifies the some of the important enzymes as well (Hayat *et al.*, 2010).

2.2.7 Cycocel

It is the common name for the 2-chloroethyl-trimethylammonium cation, $ClCH_2CH_2N^+ (CH_3)_3Cl$ (also known as chloromequat). CCC is highly water-soluble and widely used in many parts of the world in the culture of cereals, fruit and ornamental plants.

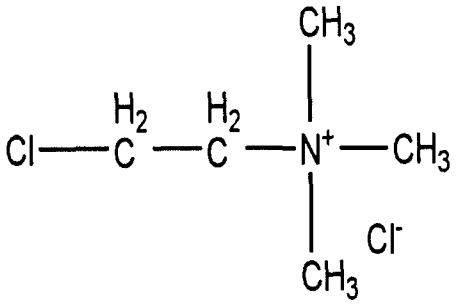

Fig. 12. Cycocel

It delays senescence in bean and lettuce. It supresses vegetative growth but increases floral growth in *Azalea*. It accelerates flowering in short day plants but not in long day plants. CCC reduces fruit set in apple but increases in grapes. It increases the tolerance capacity of plants against drought, frost and water logging (Sinha, 2013).

2.3 Effect of various PGRs on *Mentha* and other essential oil producing plants

Various PGRs have been found to stimulate growth and terpenoid biosynthesis in various aromatic plants including mint species. In the following pages, an effort has been made to review the available literature on the effect of various PGRs on the performance of mint species.

Kewalanand *et al.* (1998) studied the effect of PGRs on the growth, herbage and oil yield of *Mentha arvensis* L. (Japanese mint) var. MAS-1 and its economics therefrom. The experimental findings revealed that values for growth parameters, oil content, fresh herbage and oil yield were significantly enhanced due to application of 40 mg L^{-1} GA_3 compared with naphthalene acetic acid and the control. Increasing rates of plant growth regulators increased the menthol content in oil, highest being due to 40 mg L^{-1} GA_3. They concluded that sucker treatment method caused significant enhancement in growth parameters and yield over foliar application. However, menthol content in oil enhanced due to foliar application.

Singh *et al.* (1999) conducted an experiment on *Mentha spicata* (spearmint) var. MSS-5 to study the influence of ethrel and GA_3 on carbon metabolism, growth and its essential oil content. The data of the experiment revealed that GA_3 and ethrel treatments induced significant phenotypic changes and a decrease in chlorophyll

content, CO_2 exchange rate, and stomatal conductance compared with the control. They concluded that ethrel and GA_3 influence the partitioning of primary photosynthetic metabolites and thus modify plant growth and essential oil accumulation.

Farooqi *et al.* (2003) carried out an experiment to study the effect of Kn and CCC on growth, leaf abscission and essential oil yield of two varieties of Japanese mint (Kosi and Shivalik) and reported that herbage and oil yield increased significantly by Kn (200 ppm) in Kosi and Shivalik and by CCC (1000 ppm) in Shivalik. The result also revealed that chlorophyll content is increased due to application of Kn (100 ppm) and CCC (1000 ppm) in both varieties.

Hashmi (2004), conducting experiments on Japanese mint var. Saryu, studied the effect of two PGRs (GA_3 and Kn) on the growth, physiological and yield characteristics of the crop. The data of the experiment showed that GA_3 at 10^{-4} M and Kn at 10^{-5} M concentration proved very effective for the growth, physiological and yield characteristics.

Zheliazkov (2010) studied the effect of three plant hormones (SA, MJ, and GA_3), each at 10, 100 and 1000 mg L^{-1}, applied as a foliar spray, on biomass yields, essential oil content and oil yields of peppermint var. Black Mitcham and spearmint var. Native. Their study showed that the application of SA at 1000 mg L^{-1} increased biomass yields of both mints relative over the control, however the other treatments were not significantly different from the control. The essential oil content was higher in peppermint than in spearmint, and treatments had different effect on the two mint species. The application of MJ at 100 and 1000 mg L^{-1}, GA_3 at 10 mg L^{-1}, SA at 10 and at 100 mg L^{-1}, increased the essential oil content of peppermint, while the oil content of spearmint was not affected by treatments. None of the treatments reduced essential oil content relative to the respective controls. The overall essential oil were also differentially affected by the treatments in the two mint species. The application of MJ at 100 mg L^{-1} and SA at 100 mg L^{-1} increased essential oil yields of peppermint, while the application of SA at 1000 mg L^{-1} increased oil yields of spearmint. Treatments did not reduce essential oil content or oil yield of either mint species. This study demonstrated that plant hormones may be used as a tool for

increasing essential oil content and essential oil yields of peppermint and spearmint crops.

Sharafzadeh and Zare (2011) presented a review which revealed that the role of growth regulators on growth and secondary metabolites of some medicinal plants from Lamiaceae. They concluded that growth regulators can influence essential oil production through effects on plant growth, essential oil biosynthesis and the number of oil storage structures. On the other hand, the time of exogenous application and kind of growth regulators can affect essential oil content.

Kavina *et al.* (2011) observed the effect of different plant growth regulators and fungicide treatments on the growth characteristics of peppermint. Two PGRs GA_3 and abscissic acid (ABA) were used. ABA increased the fresh weight, dry weight, root growth, total chlorophyll, protein and amino acid content, while it decreased the stem length. GA_3 enhanced fresh and dry weights and stem length at the larger extent when compared with control.

Naeem *et al.* (2011) at Aligarh studied the effect of leaf applied four concentrations of Tria (10^{-0}, 10^{-7}, 10^{-6} and 10^{-5} M) on growth and other physiological attributes, crop yield and quality attributes and the yield and contents of active constituents (menthol, L-menthone, isomenthone and menthyl acetate) of Japanese mint var. Saryu at 100 and 120 days after planting (DAP). The data revealed that the foliar application of Tria enhanced most of the growth and other physiological attributes, crop herbage yield and the yield and content of active constituents of Japanese mint, with 10^{-6} M Tria proving optimum.

Naeem *et al.* (2012) further observed the effect of leaf applied four concentrations of 28-homobrassinolide (Hbr), i.e. 10^{-0}, 10^{-8}, 10^{-7} and 10^{-6} M on physiological attributes, herbage yield, content and yield of essential oil and active constituent (menthol, L-menthone, isomenthone and menthyl acetate) of Japanese mint (var. Saryu) at 100 and 120 DAP. The figures from this experiment revealed that the foliar application of Hbr enhanced physiological attributes, herbage yield and the yield and content of most of the active constituents of Japanese mint at both the stages, with

10^{-7} M concentration proving the best. However, the next higher concentration of Hbr (10^{-6} M) exhibited no further increase in values for the attributes studied.

Naeem *et al.* (2014) in continuation also studied the effect of irradiated sodium alginate (ISA), Tria and Hbr on physiological activities, production of essential oil and active components (menthol, L-menthone, isomenthone and menthyl acetate) of Japanese mint var. Saryu at 100 and 120 DAP. They gave seven spray-treatments, i.e. (i) control, (ii) 100 ppm ISA, (iii) 10^{-6} M Tria, (iv) 10^{-7} M Hbr, (v) 100 ppm ISA + 10^{-6} M Tria, (vi) 100 ppm ISA + 10^{-7} M Hbr, (vii) 100 ppm ISA + 10^{-6} M Tria + 10^{-7} M Hbr. Application of 100 ppm ISA + 10^{-6} M Tria + 10^{-7} M Hbr proved to be the best. The combined application resulted in the highest content and yield of essential oil over the control by 42.1 and 43.9% and 114.0 and 121.7% at 100 and 120 DAP, respectively. They concluded that the effect of the combined application was much pronounced compared with that of their individual application.

Bose *et al.* (2013) observed the effect of GA_3 and calliterpenone (CA) on growth attributes, trichomes, essential oil biosynthesis and pathway gene expression in differential manner in Japanese mint. The statistics from the above experiment revealed that the exogenous application of CA (1 µM, 10 µM or 100 µM) was found to be better in improving plant biomass and stolon yield, leaf area, branching and leaf stem ratio than that of GA_3.

Reda *et al.* (2005) studied the effect of GA_3, IBA, ascorbic acid, thiamine and nicotinamide on essential oil, phenolic components and polyphenol oxidase activity of *Thymus vulgaris* L. (thyme). They applied two concentrations of each PGR, i.e. 30 and 60 mg L^{-1}. The data revealed that the greatest oil percentage was obtained at treatment 30 mg L^{-1} of IBA or nicotinamide in the herb sampled at cutting I and the increase in oil percent was 36 and 27%, respectively, over the control. At cuttings II and III, the essential oil percentages were significantly decreased at most treatments, more so with 30 and 60 mg L^{-1} GA_3. They concluded that in all treatments, thymol was a predominant constituent (53.96-34.21%) followed by p-cymene (44.62-27.53%) and then by carvacrol (5.22-3.03%) in the oil of thyme herbage.

Kim *et al.* (2006) studied the effect of MJ on secondary metabolites of *Ocimum basilicum* L. (sweet basil). They reported that total phenolic content of the sweet basil significantly increased after 0.1 and 0.5 mM MJ treatments compared with the control not subjected to MJ.

Gharib (2007), studied the effect of SA on the growth, metabolic activities and oil content of sweet basil and *Origanum majorana* L. (marjoram). They used three concentrations of SA (10^{-5}, 10^{-4} and 10^{-3} M). The figures revealed that SA increased plant height, number of branches, nodes and leaves per plant, leaf area, fresh and dry weight of herbs, total carbohydrates, crude protein, total amino acids, free proline, photosynthetic pigments as well as microelement content and uptake up to 10^{-4} M relative to un-treated controls and decreased thereafter both in basil and marjoram. All SA treatments enhanced putrescine, spermidine as well as total polyamines contents, while reduced the level of spermine in both plants. Oil percentage and yield per plant for three cuttings also increased about two fold on a fresh weight basis with SA application at 10^{-4} M in case of basil and 10^{-3} M in marjoram relative to un-treated controls.

Singh *et al.* (2008) noted the effect of three PGRs, i.e. Kn, IAA, paclobutrazol (Pbz) on growth, flower, oil yield, and quality of *Salvia sclarea* L. (clary sage). They used Kn at 5 and 10 μL L^{-1}, IAA at 50 and 100 μL L^{-1} and Pbz at 40 and 80 μL L^{-1}. The data showed that maximum height and leaf numbers were obtained after application of 10 μL L^{-1} Kn and 50 μL L^{-1} IAA, respectively. Maximum flower and oil yield were observed after application of 40 μL L^{-1} Pbz. Application of 80 μL L^{-1} Pbz increased the linalool-linalyl acetate content of the plant about 12% higher than the untreated control.

Rowshan *et al.* (2010) investigated the effect of SA on quality and quantity of essential oil components in *Salvia macrosiphon* (wild sage). They used four concentrations of SA (0, 200 and 400 mg L^{-1}). They reported that essential oil yield was increased from 0.23% (0 mg L^{-1}) to 0.48% (400 mg L^{-1}).

Ono *et al.* (2011) conducted an experiment in green house to study the effect of PGR application on yield and composition of the essential oil of sweet basil. GA_3, ethephon

and Kn were applied at 100 mg L^{-1} at 30, 50 and 70 DAS respectively and essential oil was assessed at 90 DAS. The data revealed that plants treated with ethephon had the highest essential oil yield, while those treated with GA_3 had lowest oil yield. Treatment with Kn increased linalool and eugenol levels, whereas ethephon treatment increased eugenol content.

Rahimi *et al.* (2013) in Iran studied the effect of SA and MJ on growth and secondary metabolites in *Cuminum cyminum* L. (cumin). They observed that the lowest concentrations of SA (0.01 and 0.1 mM) resulted in significant promotion of plant height and number of branches and umbels per plant. Fruit yield and essential oil yield significantly increased by the application of 0.1 mM SA. The essential oil percentage was increased by SA and MJ application; however the increase of the essential oil was more evident by applying the SA treatments. Also in Iran, Yadegari and Shakerian (2014) conducted an experiment to study the effect of foliar application of SA and JA on essential oil of *Salvia officinalis* L. (salvia). The results showed that JA was more effective in stimulating the accumulation of α pinene, limonene, b-pinene, camphor, thymol, camphene, thujone-trans, thujone cis, 1, 8 cineole, borneol, borneol acetate, carvacrol, and caryophyllene.

2.4 Conclusion

On the basis of the above review of literature, it can be concluded that not much work has been done to compare the effect of different PGRs on the performance of peppermint. Therefore, the present experiment was conducted to fullfill the existing gap.

CHAPTER 3

MATERIALS AND METHODS

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CHAPTER 3

MATERIALS AND METHODS

To achieve the objective framed in Chapter 1, one simple randomized pot experiment was conducted on peppermint during the summer season of 2013. The experiment was conducted in a net-house of the Department of Botany, Aligarh Muslim University, Aligarh. The details of agro-climatic conditions, preparations of PGR solution, soil analyses and techniques and procedures employed are given below.

3.1 Agro-climatic conditions

Aligarh is a city of western Uttar Pradesh (North India) and is located approximately 140 km south-east of Delhi at 27°88' N latitude, 78°08' E longitude and 178 m altitude. It has a monsoon influenced humid-subtropical climate with three principal seasons from intense cold winters, monsoons to severest hot dry summers. Winter season extends from the middle of October to the end of March. The mean temperatures for December and January, the coldest months, are about 17.4° C and 10° C respectively. The extreme minimum temperature recorded for any single day is 0.5° C. The summer season prevails from April to June. The average temperatures for May and June, the hottest months are 35° C and 33° C respectively, whereas the extreme maximum record is 45.5° C. The monsoon extends from the end of June to the middle of October and the average temperature for August, the maximum rainfall occurring month, is about 29.5° C. The extreme maximum temperature record is 36° C (Fig. 1). The average annual rainfall in the district is 847.30 mm. More than 85% of the rainfall occurs during June to September and some 10% in the winter. Additional occasional rainfall during the summer is rare, sporadic, short lived and highly variable in amounts. On an average, 4% of the total rainfall occurs during this season (Fig. 2). The relative humidity of the winter season ranges between 56% and 77% with an average of 66.5%, of the summer season, between 37% and 49% with an average of 43%, and of the monsoon season, between 63% and 73% with an average of 68% (Fig. 3). Aligarh district has the same soil composition and appearances as those found generally in the plains of Uttar Pradesh. Different types of soils, such as sandy, loamy, sandy-loam and clayey-loam are found in the district.

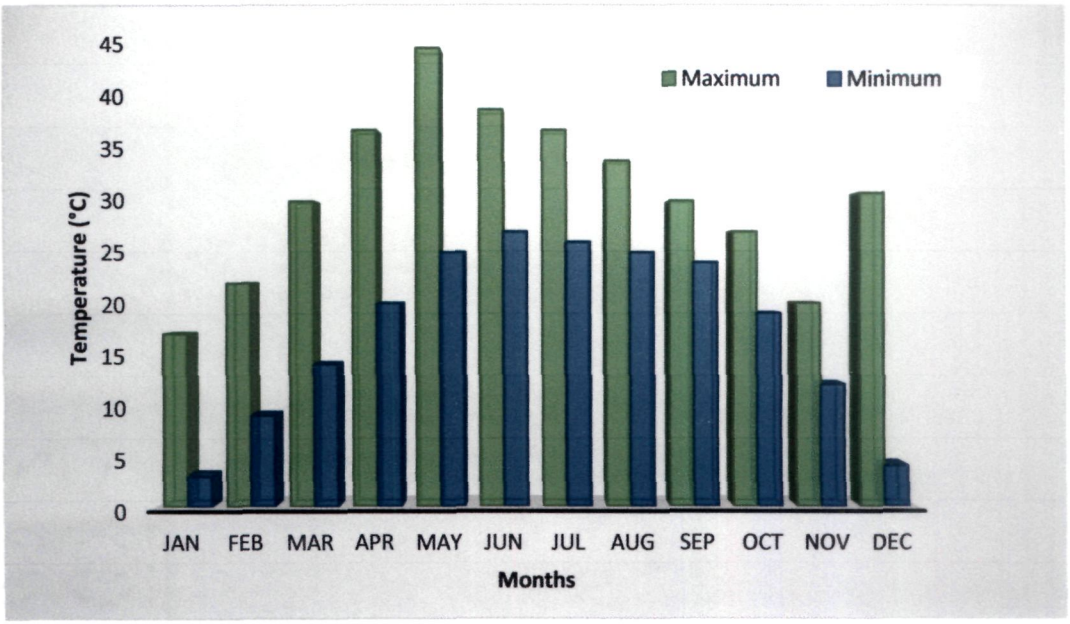

Fig. 13. Monthly temperature variation at Aligarh

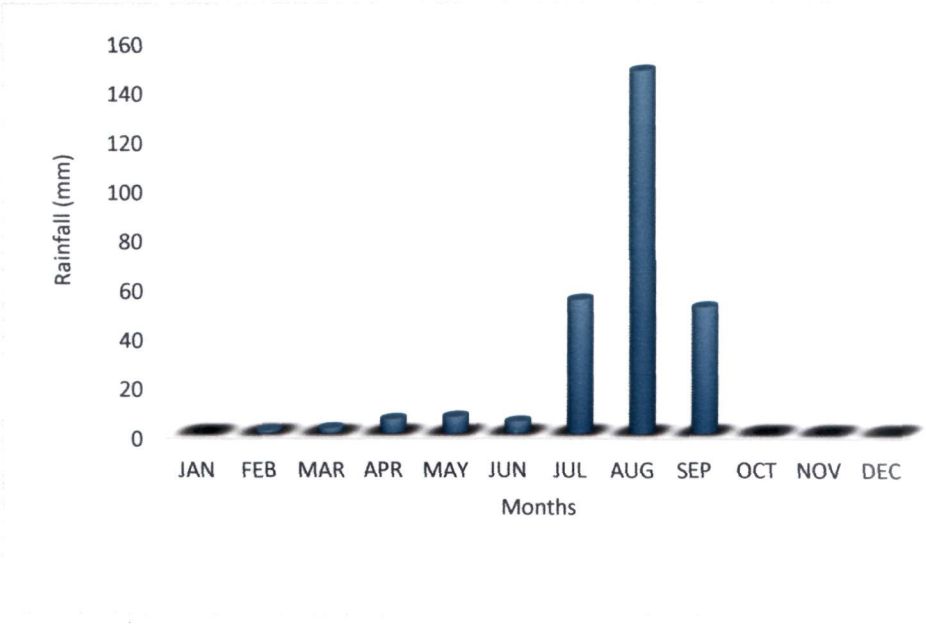

Fig. 14. Average monthly rainfall at Aligarh

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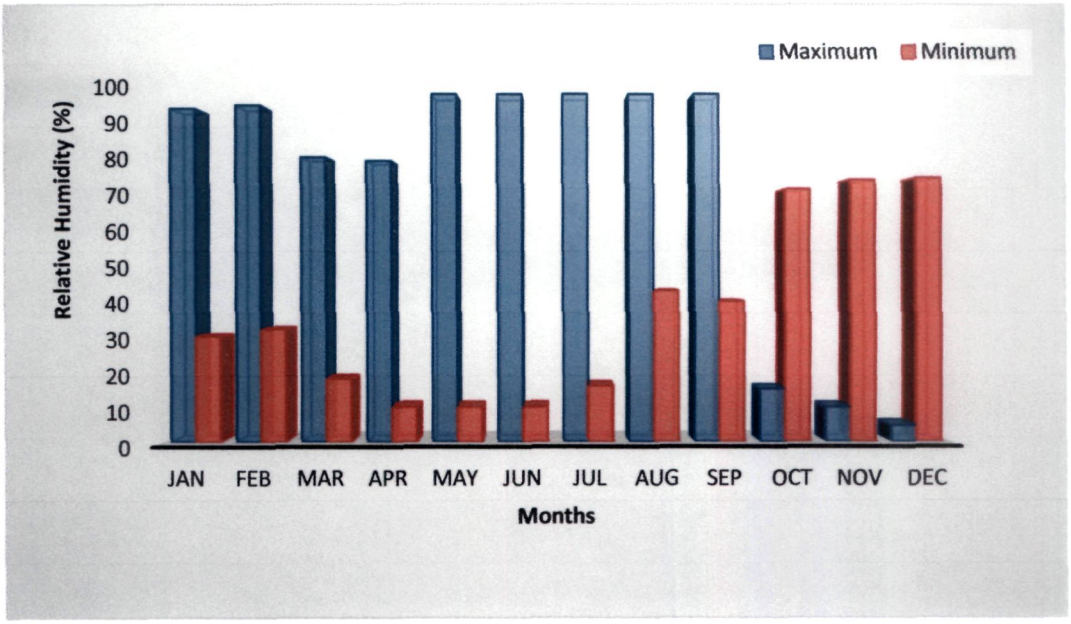

Fig. 15. Monthly relative humidity variation at Aligarh

3.2 Filling of pots

The soil was collected from a farmer's field up to the depth of about 10-15 cm. After removing weeds and undesirable particles, the soil was mixed uniformly with the farmyard manure in the ratio of 5:1. Each earthen pot (25 cm diameter x 25 cm height) was filled with at the rate of 5 kg homogenous mixture.

3.3 Soil analysis

Before sowing, soil samples were collected randomly from different experimental pots for the analysis of the soil characteristics. The observed physico-chemical properties of the soil are given in Table1.

Table 1. Physico-chemical properties of soil

S. No.	Soil characteristics	Values
1	Texture	Sandy loam
2	pH (1:2)	7.8
3	Electrical conductivity at 1:2 (dSm^{-1})	0.57
4	Available N (mg kg^{-1} soil)	98.5
5	Available P (mg kg^{-1} soil)	7.15
6	Available K (mg kg^{-1} soil)	141.8

3.4 Peppermint suckers

The healthy suckers of peppermint were obtained from the Central Institute of Medicinal and Aromatic Plants, Lucknow (India).

3.5 Pot experiment

One pot experiment was conducted on peppermint during the summer season of 2013. The soil was maintained at sufficient moisture to ensure optimum growth of plants. The aim of the experiment was to select the most suited one among nine PGRs for the optimum performance of peppermint grown with the recommended basal dose of 40 mg N + 40 mg P + 40 mg K kg^{-1} soil (N_{40} P_{40} K_{40}) under agro-climatic conditions of western Uttar Pradesh. N, P and K were applied in the form of urea, single superphosphate and muriate of potash, respectively at the time of transplanting. The experiment was conducted according to a simple randomized design. Each treatment was replicated five times. One sucker per pot was transplanted on 14th February, 2013. Five sprays of dilute solution (5×10^{-6} M) each of nine PGRs were applied to the foliage of plants using a hand sprayer at the interval of 5d with the first spray giving at 90 DAT. The PGRs included GA_3, IAA, IBA, BAP, Kn, SA, MJ, Tria and CCC. The control plants were sprayed with double distilled water (DDW). In all, there were ten treatments. The plants were kept free from weeds and were irrigated as and when required. The standard cultural practices were adopted for growing the crop.

3.6 Preparation of stock solutions of PGR

Prior to the foliar treatments, 100 milli-litre (mL) stock solutions of PGR were prepared at 10^{-3} M concentration. The required amount of IAA, IBA, Kn and BAP was dissolved in 10 mL 1N sodium hydroxide (NaOH) separately. The final volume was made 100 mL using DDW. The required quantity of GA_3, MJ and CCC was dissolved in 10 mL ethyl alcohol separately and the final volume was made 100 mL with DDW. The required quantity of SA and Tria was dissolved in 10 mL DDW separately and the final volume was made 100 mL using DDW. The required concentration at 5 x 10^{-6} M of each PGR was prepared by diluting the stock solution using DDW.

Table 2. Scheme of treatments

PGR *treatments (5 x 10^{-6} M)* (spray)	Remark
Water (Control)	Deionized water
GA_3	Gibberellic acid
IAA	Indole acetic acid
IBA	Indole butyric acid
BAP	Benzyl amino purine
Kn	Kinetin
Tria	Triacontanol
MJ	Methyl jasmonate
SA	Salicylic acid
CCC	Cycocel

N.B: A uniform recommended basal dose of $N_{40}P_{40}K_{40}$ was applied at transplanting

Replicates : 5

PGR treatments : 10

Design : Simple randomized

3.7 Sampling techniques

Five plants from each treatment were uprooted carefully and washed under tap water to remove all adhering foreign particles and brought to laboratory for growth, biochemical and quality analyses. Growth characteristics and physiological and biochemical parameters were studied at 120 DAT, and yield and quality parameters, at harvest. The following parameters were studied:

3.8 Growth characters

3.8.1 Fresh weight per plant

3.8.2 Dry weight per plant

3.9 Physio-biochemical parameters

3.9.1 Chlorophyll content

3.9.2 Carotenoid content

3.9.3 Nitrate reductase activity

3.9.4 Carbonic anhydrase activity

3.9.5 Leaf nitrogen, phosphorus and potassium content

3.10 Yield and quality characteristics

3.10.1 Essential oil content in leaves

3.10.2 Essential oil yield per plant

3.10.3 Menthol, menthone and menthyl acetate contents in the oil

3.10.4 Menthol, menthone and menthyl acetate contents yield per plant

3.8 Growth characters

3.8.1 Fresh weight per plant

The plants were washed with tap water to remove adhering foreign particles, followed by surface drying using blotting paper and fresh weight of the plant was recorded using an electronic balance.

3.8.2 Dry weight per plant

The plants were dried in a hot-air oven at 80° C for 48 h and the dry weight per plant was recorded using an electronic balance.

3.9 Physio-biochemical parameters

3.9.1 Chlorophyll content

The total chlorophyll content was estimated by the method of Mackinney (1941). One hundred mg fresh leaves were ground in 10 mL 80% acetone (Appendix), using a mortar and pestle. The suspension was decanted and filtered through a Whatman filter paper No.1 into a test tube through Buchner funnel. The optical density (OD) of the solution was read at 645 and 663 nm using a spectrophotometer (Spectronic UV-1700, Shimadzu, Japan). The total chlorophyll content was calculated using the following formula:

$$\text{Total chlorophyll content} = 20.2\ (OD\ 645) + 8.02\ (OD\ 663) \times \frac{V}{W \times 1000}\ mg\ g^{-1}\ FW$$

Where,

V = Final volume of the leaf extract in 80% acetone

W = Fresh weight of leaf tissue (g)

d = Length of light path (1 cm)

3.9.2 Carotenoid content

The total carotenoid content was estimated by adopting the method of MaClachlan and Zalik (1963). The working leaf extract prepared for chlorophyll estimation was also used for carotenoid estimation. The OD of the solution was read at 480 and 510 nm using the spectrophotometer. Total carotenoid content was determined by the following formula:

$$\text{Total carotenoid content} = 7.6\ (OD\ 480) - 1.49\ (OD\ 510) \times \frac{V}{d \times W \times 1000}\ mg\ g^{-1}\ FW$$

Where,

V = Final volume of the leaf extract in 80% acetone

W = Fresh weight of leaf tissue (g)

d = Length of light path (1 cm)

3.9.3 Nitrate reductase activity

The enzyme nitrate reductase (NR) activity was estimated by the intact tissue method of Jaworski (1971) based on the reduction of nitrate to nitrite as per the following biochemical reaction:

$$NO_3^- + NADH + H^+ \xrightarrow{\text{Nitrate reductase}} NO_2^- + NAD^+ + H_2O$$

The nitrite formed in the reaction was determined spectrophotometrically. The leaves of each sample were cut into small pieces (1 cm^2). These chopped leaves were weighed at 200 mg and transferred into a separate plastic vial. Into this vial, 2.5 mL phosphate buffer (pH 7.5, Appendix) and 0.5 mL 0.2 M potassium nitrate solution (Appendix) were added followed by the addition of 2.5 mL 5% isopropanol (Appendix). Finally, two drops of chloramphenicol solution were added to avoid bacterial growth in the medium. The vial was incubated in a BOD incubator for 2h at 30±2°C in dark. Incubated mixture was taken at 0.4 mL into a test tube followed by the addition of 0.3 mL each 1% sulphanilamide solution (Appendix) and 0.02% N-1-nepthyl-ethylenediamine-dihydrochloride (NED-HCl) solution (Appendix). The test tube was left for 20 min at room temperature for maximum colour development. The mixture was diluted to 5 mL with DDW. The OD was read at 540 nm on the spectrophotometer. A blank was run simultaneously with each sample. The OD of the sample was compared with a standard curve and NR activity was expressed as nM NO_2^- kg^{-1} (leaf fresh matter) s^{-1}.

3.9.3.1 Standard curve

Sodium nitrite ($NaNO_2$) at 30 mg was dissolved in 100 mL DDW. Of this solution, 0.8 mL was taken into a 100 mL volumetric flask. The volume was made up to 100 mL using DDW. From this diluted solution, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mL were taken into separate test tubes. Into each test tube, 0.3 mL each 1% sulphanilamide and 0.02% NED-HCl were added. The solution was diluted to 5 mL with DDW and OD was read at 540 nm on the spectrophotometer, using a blank. A standard curve was plotted using the selected concentrations of pure $NaNO_2$ versus OD of the solution.

3.9.4 Carbonic anhydrase activity

The enzyme carbonic anhydrase (CA) catalyses the reversible hydration of carbon dioxide (CO_2) to give the bicarbonate ion (HCO^{-3}).

$$H_2O + CO_2 \xrightleftharpoons{\text{Carbonic anhydrase}} H^+ + HCO^{-3}$$

The activity of the enzyme was determined by the method of Dwivedi and Randhawa (1974). The leaves were cut into small pieces (1 cm^2) at temperature below 25° C. After mixing them, 200 mg leaf pieces were weighed and cut further into smaller pieces keeping them in 10 mL 0.2 M aqueous cystein solution (Appendix) in a Petridish at 0° C to 4° C for 20 min. The solution adhering on cut surfaces of the leaf pieces was then removed with the help of a blotting paper followed by their transfer immediately into a test tube containing 4 mL phosphate buffer of pH 6.8 (Appendix). Into this, 4 mL 0.2 M sodium bicarbonate ($NaHCO_3$) in 0.02 M sodium hydroxide (NaOH) solution (Appendix) and 0.2 mL 0.002% bromothymol blue indicator (Appendix) were added. After shaking, the tube was kept at 0° C to 4° C for 20 min. Carbon dioxide liberated during catalytic action of enzyme on $NaHCO_3$ was estimated by titrating the reaction mixture against 0.05 N HCl (Appendix), using methyl red (Appendix) as an internal indicator. A control reaction mixture (without leaf pieces) was also titrated against 0.05 N HCl. The difference of sample reading and control reading was noted for further calculation of enzyme activity.

The activity of the enzyme was determined by the following formula:

$$\frac{V \times 22 \times N}{W} \mu M\ CO_2\ mg^{-1}\ 20\ min^{-1}$$

V = difference in volume (mL) of hydrochloric acid used in the control and sample

22 = equivalent weight of CO_2

N = normality of HCl (36.5)

W = weight (mg) of leaves used

Finally, the activity of the enzyme was expressed in terms of mM CO_2 kg^{-1} (leaf fresh matter) s^{-1}.

3.9.5 Leaf nitrogen, phosphorus and potassium contents

The leaf samples were dried in hot-air oven at 80° C for 48 hours. Dried leaves were finely powdered and the powder thus obtained was sieved. The powder was labelled and stored in separate small polythene bags for the analysis.

The oven-dried leaf powder at 100 mg was transferred into a 50 mL Kjeldahl flask into which 2 mL analytical reagent grade concentrated sulphuric acid (H_2SO_4) was added. The flask was heated on a temperature controlled assembly at 80° C for about 2h to allow complete reduction of nitrate present in the plant material by organic matter itself. As a result, the content of the flask turned black. After cooling the flask for about 15 min at room temperature, 0.5 mL 30% hydrogen peroxide (H_2O_2) was added drop by drop and the solution was heated again till the colour of the solution changed from black to light yellow. Again, after cooling for about 30 min, 3-4 drops of 30% H_2O_2 were added, followed by heating for another 5 min. The addition of 30% H_2O_2 followed by heating was repeated until the content of the flask became colourless. The peroxide-digested material was transferred from the Kjeldahl flask into a 100 mL volumetric flask with three washings each with 5 mL DDW. The volume of the volumetric flask was made up to the mark with DDW and the H_2SO_4-H_2O_2 digested material was used to estimate N, P and K contents. The details of methods used for the analysis of these elements are given below separately.

3.9.5.1 Nitrogen

N content was estimated according to the method of Lindner (1944). A 10 mL H_2SO_4-H_2O_2 digested material was taken into a 50 mL volumetric flask. Into which, 2 mL 2.5 N sodium hydroxide (Appendix) and 1 mL 10% sodium silicate solution (Appendix) were added to neutralize the excess of acid and to prevent turbidity respectively. The volume was made up to the mark with DDW. Into a 10 mL graduated test tube, 5 mL this solution was taken and 0.5 mL Nessler's reagent was added. The contents of the test tube were allowed to stand for 5 min for maximum colour development. The solution was transferred into a spectrophotometric tube and the OD of the solution was read on the spectrophotometer at 525 nm. A blank was also run simultaneously. The reading of each sample was compared with the standard calibration curve and N content was expressed in the terms of percentage on dry weight basis.

3.9.5.1.1 Standard curve

Pure ammonium sulphate at 50 mg was dissolved in a sufficient volume of DDW in a 1000 mL volumetric flask and the final volume was made up to 1000 mL with DDW. From this solution, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL solution was pipetted into ten separate test tubes. The solution in each test tube was diluted to 5 mL with DDW. Into each test tube, 0.5 mL Nessler's regent was added. After 5 min, the OD was read at 525 nm, using the spectrophotometer. A blank was also run side by side. Standard curve was plotted using known graded concentrations of ammonium sulphate solution versus OD.

3.9.5.1.2 Phosphorus

The method of Fiske and Subba Row (1925) was used to estimate the total P in the H_2SO_4-H_2O_2 digested material. The digested material at 5 ml was taken into a 10 mL graduated test tube and 1 mL 2.5% molybdic acid (Appendix) was added carefully, followed by addition of 0.4 mL 1-amino-2-naphthol-4 sulphonic acid (Appendix). When the colour turned blue, the volume was made up to 10 mL with DDW. The solution was shaken for 5 min and was then transferred into a spectrophotometric tube. The OD of the solution was read on the spectrophotometer at 620 nm. A blank was also run side by side. The reading of each sample was compared with the standard curve and P content was expressed in terms of percentage on dry weight basis

3.9.5.2.1 Standard curve

Pure potassium dihydrogen orthophosphate at 350 mg was dissolved in sufficient DDW in a 1000 mL volumetric flask into which 10 mL 10 N H_2SO_4 was added and the final volume was made up to 1000 mL with DDW. From this solution, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL was taken into ten separate test tubes. The solution in each test tube was diluted to 10 mL with DDW. Into each test tube, 1 mL molybdic acid and 0.4 mL 1-amino-2-naphthol-4-sulphonic acid were added. After 5 min, the OD of the solution was read at 620 nm. A blank was also run simultaneously. The standard curve was plotted using different dilutions of potassium dihydrogen orthophosphate versus OD.

3.9.5.3 Potassium

K content was analyzed using flame-photometer (Hald, 1946). Basically, in this device, the solution (peroxide-digested material) is discharged through an atomizer in the form of a fine mist into a chamber, where it is drawn into a flame. Combustion of the elements produces light of a particular wavelength (λ max for K = 767 nm, i.e. violet). The light produced was conducted through the appropriate filter to impinge upon a photoelectric cell that activates a galvanometer. The air was supplied through an air pump and liquid petroleum gas was used for combustion. The chimney of the equipment was removed and the gas was ignited using an electric lighter. The final pressure of the two gases was adjusted to 15 lb $inch^{-1}$. When the flame formed sharp blue cones, the compatible filter was set and DDW (blank) introduced (using a beaker) and galvanometer was set to zero. Then the standard solution of the element (10 ppm) allowed to suck through a capillary tube and the galvanometer was adjusted to the 100 position by using the amplifier. Unless the 0 and 100 points are maintained on successive readings, the gas pressure, air-pressure or both were adjusted to bring about a stable position. For determination of K content, 10 mL aliquot of the H_2SO_4-H_2O_2 digested material was allowed to read on a flame photometer (Model: C 150, AIMIL, India) using the filter for K. A blank was run side by side. The reading of each sample was compared with the standard curve plotted and K was expressed in terms of percentage on dry weight basis.

3.9.5.3.1 Standard curve

Potassium chloride (KCl) at 1.91 g was dissolved in 50 mL DDW in a 100 mL volumetric flask followed by dilution to 100 mL with DDW. 1 mL of this solution was diluted to 1000 mL. The resultant stock solution would contain 10 ppm K, from which 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mL aliquots were transferred into 10 different vials. The solution in each vial was diluted to 10 mL. The diluted solution of each vial was run separately. A blank was also run with each set of determination. The standard curve was prepared for different dilutions of KCl solution versus reading on the scale of the galvanometer.

3.10 Yield and quality characteristics

3.10.1 Essential oil content in leaves

The essential oil of peppermint was extracted using a Clevenger's apparatus. This is a continuous distillation apparatus in which the separated volatile oil is caught in a trap. The apparatus consists of distillation flask with a heating mantle, still head, graduated measuring tube, levelling tube and return tube together with a condenser fitted in one piece.

The leaves were chopped into small pieces in order to get maximum quantity of oil. These chopped leaves were transferred into the flask containing DDW. The flask then connected to the still head of Clevenger's apparatus. Before heating the distillation flask, water was run into the graduated receiver keeping the tap opened until the water overflowed. The contents of the flask were then heated using a heating mantle. The distillation was continued at a rate which kept lower end of the condenser cool by the continuous supply of tap water with the help of rubber tubes. After sometime, steam was formed in the distillation flask. The mixture of water vapour and essential oil passed into the condenser. As the distillation proceeded, the distillate collected in the graduated part of the receiver. The oil being lighter than water and insoluble in it, floated on the top of the receiver. At the end of 3 h, heating was discontinued and the apparatus was allowed to cool for 20 min. As soon as entire quantity of oil head entered the graduated part of the receiver, the volume was recorded. The measured amount of essential oil was taken to be the content of essential oil in leaf sample. The content of essential oil was expressed as a percentage on v/w basis.

3.10.2 Essential oil yield per plant

The essential oil yield per plant was computed on the basis of the essential oil percentage and fresh weight per plant.

3.10.3 Menthol, menthone and menthyl acetate contents in the oil

The menthol, menthone and menthyl acetate contents were estimated in the essential oil of peppermint using Gas Liquid Chromatography (GLC). Separation was performed by elution method in which a stream of carrier gas was passed through the column. A sample of essential oil was injected into the carrier gas as a plug of vapour which was swept into the head of the packed chromatographic column. When in the gas phase the components were moved towards the column outlet, but they were

selectively retarded by the stationary phase. Consequently, the entire components passed through the column at varying speeds and emerged in the inverse order of their retention by the column materials. Upon emerging from the column, the gaseous phase immediately entered a detector attached to the column. Here the individual component registered a series of signal which appeared as succession of peak above a base line on the chromatogram. The area under the peak was a quantitative indication of the component. The time lapse between injection and emergence of the peak (retention time) served to identify it. Menthol, menthone and menthyl acetate contents were calculated by knowing their peak areas given in their chromatogram. The contents of menthol, menthone and menthyl acetate were obtained when their peak areas were divided by the total peak areas of all components of their respective chromatograms and multiplied by 100 to determine their percentage. The conditions used for the estimation of menthol, menthone and menthyl acetate contents in the oil by GLC were as follows:

GLC, Nucon 5700, New Delhi, India was equipped with an AT-1000 stainless steel column, a flame ionization detector and an integrator. N was used as the carrier gas. The flow rates of N, H and O were maintained at 0.5, 0.5 and 5 mL s^{-1}, respectively. The temperature of GLC was throughout maintained (detector temperature: 250° C; oven temperature: 160° C; injector temperature: 250° C). The sample size was 2 µl invariably.

3.10.4 Menthol, menthone and menthyl acetate yields per plant

Menthol, menthone and menthyl acetate yields per plant were computed on the basis of their percentage and oil yield per plant.

3.11 Statistical analysis

The experimental data were statistically analysed using the analysis of variance techniques according to Gomez and Gomez (1984). In applying the 'F' test, the error due to replicate was also determined. When 'F' value was found to be significant at the level of 5% probability, the least significant difference (LSD) was calculated. The model of analysis of variance for the design employed is given in Table 3.

Table 3. Model of the analysis of variance (Simple randomized design)

Source of variation	DF	SS	MSS	F
Replicates	4			
Treatments	9			
Error	36			
Total	49			

CHAPTER 4

EXPERIMENTAL RESULTS

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CHAPTER 4

RESULTS

The important results of the experiment are described briefly in this chapter. In the simple randomized pot experiment, the effect of foliar application each of nine PGRs (5×10^{-6} M) was studied on growth, physio-biochemical and yield parameters of peppermint grown with a basal dose of N, P and K. The details of results (Tables 4-11) are briefly described below.

4.1 Growth characters

The effect of foliar application of PGRs was found to be significant on growth characters, namely plant fresh weight per plant and dry weight per plant studied at 120 DAT. The salient features of data (Table 4) are given below.

4.1.1 Fresh weight per plant

The foliar application of SA produced maximum fresh weight per plant, however its effect was equal to that of Kn. Spray application of SA increased the fresh weight per plant by 61.05% over the water-sprayed control (Table 4).

4.1.2 Dry weight per plant

The spray of SA produced maximum dry weight per plant. The effect of SA spray was followed by that of Kn. The spray of SA exceeded the control by 169.18% (Table 4).

4.2 Physio-biochemical parameters

The effect of foliar spray of PGRs was found to be significant on physio-biochemical parameters, namely chlorophyll content, carotenoid content, NR activity, CA activity and leaf N, P and K contents studied at 120 DAT (Tables 5-7). The salient features of data (Tables 5-7) are given below.

4.2.1 Chlorophyll content

The foliar application of SA resulted in the highest value for chlorophyll content. Its effect was followed by that of MJ. The spray treatment of SA increased the chlorophyll content by 40.07% over the water-sprayed control (Table 5).

4.2.2 Carotenoid content

The spray of SA gave the maximum carotenoid content. Its effect was followed by that of MJ and Tria. The application of SA registered 39.41% higher value for carotenoid content than the water-sprayed control (Table 5).

4.2.3 Nitrate reductase activity

The foliar spray of Tria proved the best for NR activity. Its effect was followed by that of GA_3. The spray of Tria exceeded the water-sprayed control by 20.08% (Table 6).

4.2.4 Carbonic anhydrase activity

The foliar application of SA gave the maximum value for CA activity. Its effect was followed by that of Tria. The spray of SA enhanced the CA activity by 15.97% over the water-sprayed control (Table 6).

4.2.5 Leaf nitrogen content

The spray of SA gave the highest value for leaf N content. Its effect was followed by that of MJ. The spray treatment of SA increased leaf N content by 76.47% over the water-sprayed control (Table 7).

4.2.6 Leaf phosphorus content

The spray of Kn resulted in highest value for leaf P content. Its effect was followed by that of BAP and GA_3. The spray treatment of Kn increased leaf P content by 76.19% over the water-sprayed control (Table 7).

4.2.7 Leaf potassium content

The spray treatment of Kn resulted in the highest value for leaf K content. Its effect was followed by that of GA_3 and IAA. The spray of Kn increased leaf N content by 62.37% over the water-sprayed control (Table 7).

4.3 Yield and quality characteristics

The effect of foliar spray of PGRs was found to be significant on yield and quality characteristics, namely essential oil content in the leaves, essential oil yield per plant, menthol content in the oil, menthol yield per plant, menthone content in the oil, menthone yield per plant, menthyl acetate content in the oil, menthyl acetate yield per

plant, studied at 120 DAT (Table 8-11). The salient features of data (Tables 8-11) are given below.

4.3.1 Essential oil content in leaves

The foliar application of SA resulted in the highest value for the oil content in leaves. Its effect was followed by that of CCC. The spray treatment of SA exceeded the oil content by 70.14% over the water-sprayed control (Table 8).

4.3.2 Essential oil yield per plant

The foliar application of SA resulted in highest value for essential oil yield per plant. Its effect was followed by that of MJ. The spray treatment of SA increased the essential oil yield per plant by 179.73% over the water-sprayed control (Table 8).

4.3.3 Menthol content in the oil

The foliar spray of SA registered the maximum value for menthol content in the oil. Its effect was followed by that of GA_3. The spray treatment of SA increased the menthol content by 27.05% over the water-sprayed control (Table 9).

4.3.4 Menthol yield per plant

The foliar spray of SA proved the best for menthol yield per plant. Its effect was followed by that of MJ. The spray treatment of SA improved menthol yield per plant by 252.04% over the water-sprayed control (Table 9).

4.3.5 Menthone content in the oil

The spray treatment of BAP gave the maximum value for menthone content in the oil. Its effect was followed by that of IAA. The foliar application of BAP increased menthone content in the oil by 25.54% over the water-sprayed control (Table 10).

4.3.6 Menthone yield per plant

The spray treatment of Tria exhibited the maximum value for menthone yield per plant. Its effect was followed by that of SA. The foliar application of Tria increased the menthone yield per plant by 329.55% over the water-sprayed control (Table 10).

4.3.7 Menthyl acetate content in the oil

The spray treatment of GA_3 gave the highest value for menthyl acetate content in the oil. Its effect was followed by that of MJ and Kn. The application of GA_3 increased the menthyl acetate content by 56.84% over the water-sprayed control (Table 11).

4.3.8 Menthyl acetate yield per plant

The foliar application of SA surpassed other treatments. Its effect was followed by that of MJ. The foliar treatment of SA improved menthyl acetate yield per plant by 191.67% over the water-sprayed control (Table 11).

Table 4. Effect of PGR spray treatments on fresh and dry weight per plant of peppermint

PGR spray treatments (5×10^{-6} M)	Plant fresh weight (g)	Plant dry weight (g)
Water (Control)	77.520	16.140
GA_3	85.870	19.440
Kn	121.840	39.486
IAA	96.914	24.438
IBA	93.858	21.528
BAP	100.264	30.656
SA	124.846	43.446
Tria	114.532	35.604
CCC	80.632	17.632
MJ	111.604	35.450
LSD at 5%	4.3545	1.4106

Table 5. Effect of PGR spray treatments on chlorophyll and carotenoid contents of peppermint

PGR spray treatments (5×10^{-6} M)	Chlorophyll content (mg g^{-1} FW)	Carotenoid content (mg g^{-1} FW)
Water (Control)	0.891	0.340
GA_3	0.858	0.312
Kn	0.926	0.406
IAA	0.871	0.384
IBA	0.888	0.368
BAP	1.095	0.394
SA	1.248	0.474
Tria	1.110	0.442
CCC	1.082	0.406
MJ	1.121	0.444
LSD at 5%	0.0040	0.0153

Table 6. Effect of PGR treatments on nitrate reductase and carbonic anhydrase activities of peppermint

PGR spray treatments (5×10^{-6} M)	Nitrate reductase activity [nM NO_2^- kg^{-1} (leaf fresh matter) s^{-1}]	Carbonic anhydrase activity [mM CO_2 kg^{-1} (leaf fresh matter) s^{-1}]
Water (Control)	239.116	233.848
GA_3	282.696	244.694
Kn	269.118	261.266
IAA	249.888	236.698
IBA	247.642	242.964
BAP	263.398	252.780
SA	277.728	271.190
Tria	287.126	265.568
CCC	258.142	232.154
MJ	254.404	254.476
LSD at 5%	2.2011	2.0359

Table 7. Effect of PGR treatments on leaf nitrogen, phosphorus, and potassium contents of peppermint

PGR spray treatments (5×10^{-6} M)	Leaf N Content (%)	Leaf P Content (%)	Leaf K Content (%)
Water (Control)	1.360	0.294	0.946
GA_3	1.760	0.440	1.492
Kn	2.254	0.518	1.536
IAA	1.462	0.408	1.478
IBA	1.378	0.370	1.440
BAP	1.952	0.462	1.346
SA	2.400	0.258	1.384
Tria	2.202	0.412	1.470
CCC	1.560	0.338	1.226
MJ	2.366	0.418	1.172
LSD at 5%	0.0151	0.0373	0.0168

Table 8. Effect of PGR treatments on essential oil content and essential oil yield per plant of peppermint

PGR spray treatments (5×10^{-6} M)	Essential oil content in leaves (%)	Essential oil yield per plant (ml)
Water (Control)	0.556	0.296
GA_3	0.348	0.208
Kn	0.436	0.376
IAA	0.388	0.260
IBA	0.374	0.238
BAP	0.408	0.290
SA	0.946	0.828
Tria	0.642	0.512
CCC	0.838	0.488
MJ	0.756	0.588
LSD at 5%	0.0120	0.0076

Table 9. Effect of PGR treatments on menthol content in the oil and menthol oil yield per plant of peppermint

PGR spray treatments (5×10^{-6} M)	Menthol content in the oil (%)	Menthol yield per plant (ml)
Water (Control)	32.782	0.098
GA_3	40.880	0.086
Kn	37.872	0.144
IAA	38.986	0.105
IBA	39.380	0.094
BAP	35.262	0.102
SA	41.650	0.345
Tria	37.130	0.189
CCC	33.072	0.161
MJ	36.580	0.216
LSD at 5%	0.0621	0.0012

Table 10. Effect of PGR treatments on Menthone content in the oil and Menthone yield per plant of peppermint

PGR spray treatments (5×10^{-6} M)	Menthone content in oil (%)	Menthone yield (ml per plant)
Water (Control)	14.920	0.044
GA_3	14.160	0.030
Kn	15.462	0.060
IAA	17.430	0.047
IBA	15.860	0.037
BAP	18.730	0.054
SA	12.850	0.107
Tria	16.062	0.189
CCC	16.580	0.083
MJ	16.290	0.095
LSD at 5%	0.0407	0.0017

Table 11. Effect of PGR treatments on Menthyl acetate content in oil and yield per plant of peppermint

PGR spray treatments (5×10^{-6} M)	Menthyl acetate content in oil (%)	Menthyl acetate yield (ml per plant)
Water (Control)	4.208	0.013
GA_3	6.600	0.014
Kn	4.830	0.018
IAA	4.790	0.012
IBA	3.495	0.008
BAP	3.671	0.012
SA	4.210	0.035
Tria	4.497	0.022
CCC	4.610	0.023
MJ	4.986	0.029
LSD at 5%	0.1818	0.0009

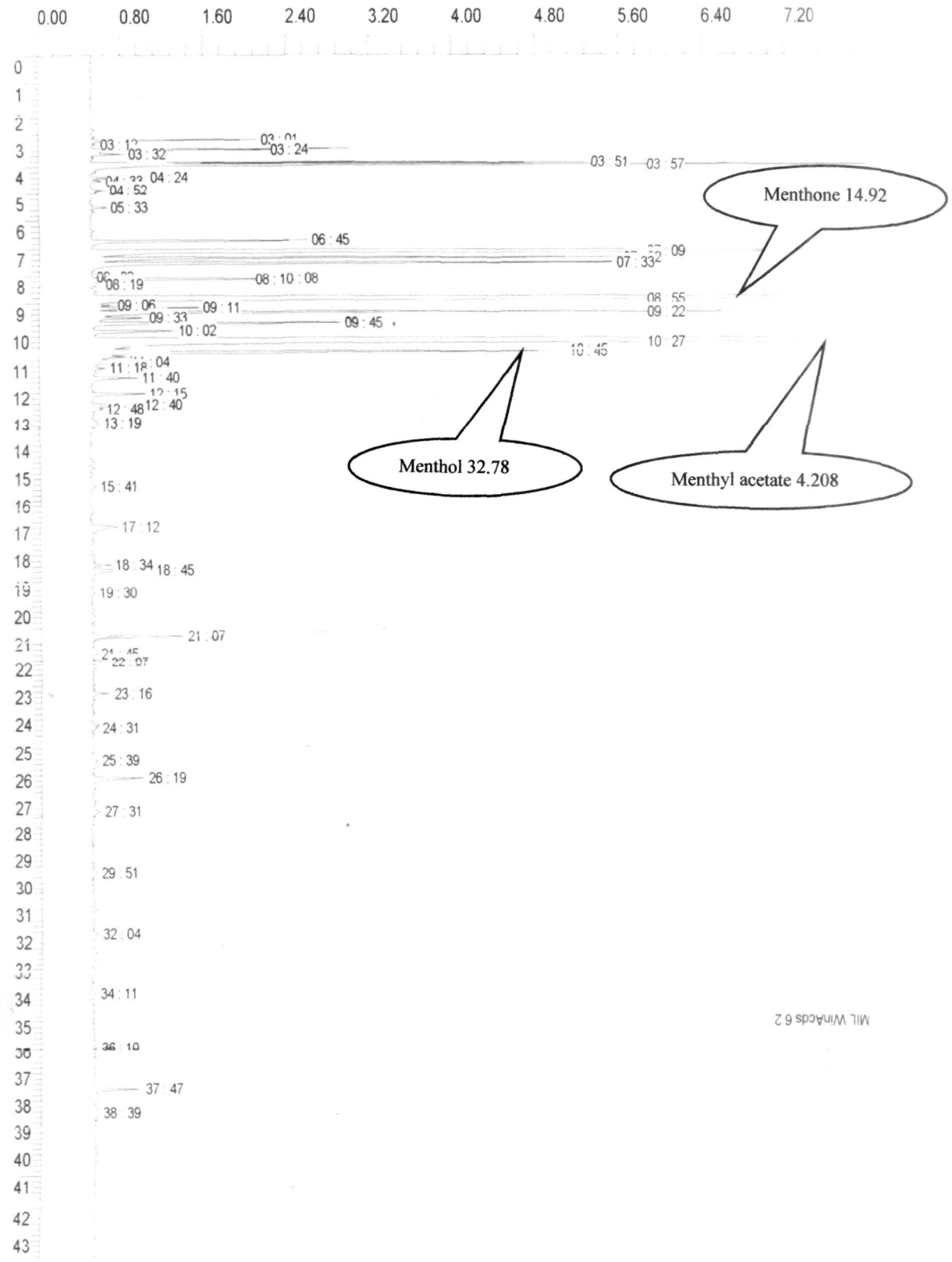

Fig. 13. GLC chromatogram of essential oil of *Mentha piperita* oil (Water treatment)

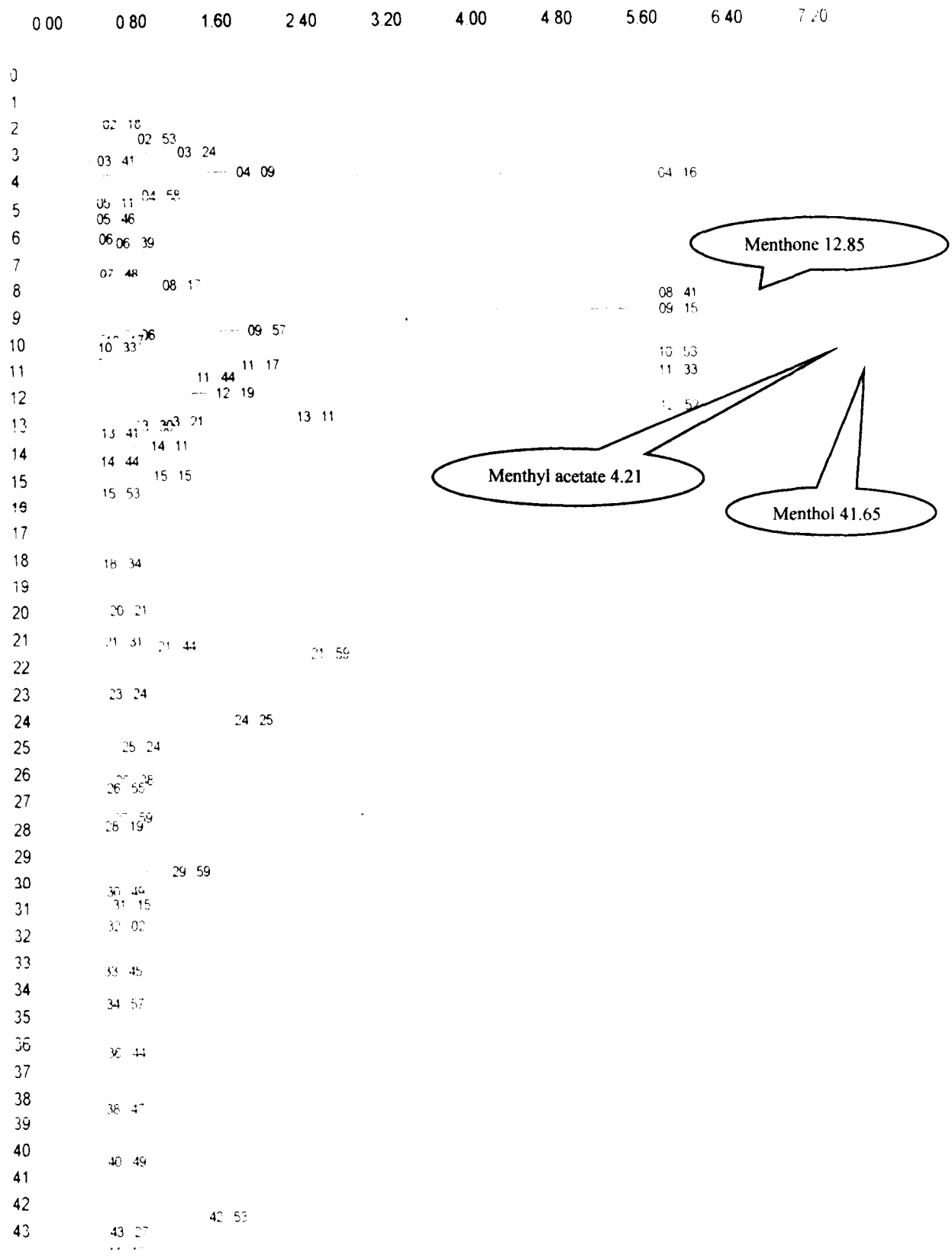

Fig. 14. GLC chromatogram of essential oil of *Mentha piperita* oil (salicylic acid treatment)

CHAPTER 5

DISCUSSION

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CHAPTER 5

DISCUSSION

Plant growth and development is a complex process and determined by several endogenous and exogenous factors. Among these factors PGRs play a vital role in regulating growth and development of plants. Various PGR show specialized effects on cell membrane properties and the transport of substances according to their physiological functions. For example, auxins increase the extensibility of the cell wall rapidly by inducing proton extrusion which acidifies the cell wall and increases cell extension (Taiz and Zeiger, 2010). Gibberellins bind to the receptor protein in the membrane of the target cell and the carrier-hormone complex moves to the nucleus and alters nucleic acid directing enzyme synthesis (Sinha, 2013). Cytokinins act either by increasing nucleic acid and protein synthesis or by involvement of a receptor protein and a second messenger. SA is involved in the regulation of the redox potential in cells. The SA level correlates with the H_2O_2 content in leaf tissues, which provides optimal redox homeostasis and regulates photosynthetic processes and trans-membrane transport under biotic and abiotic stresses (Yang *et al.*, 2004; Mateo *et al.*, 2006). Triacontanol elicits the second messenger, L(+)-adenosine. L(+)-adenosine triggers a rapidly transmitted signal within whole plants that results in a transient increase in apoplastic ion concentration within stem tissue (Ries *et al.*, 1993). CCC blocks the cyclases copalyl-diphosphate synthase and ent-kaurene synthase involved in the early steps of GA metabolism (Rademacher, 2000).

The role of PGRs in optimizing the yield and quality of various economical parts of crop plants is an established fact. PGRs are known to modify growth by increasing or decreasing the morphological traits such as plant height, number of leaves, number of branches etc. and also optimize the yield characteristics of the plant by bringing necessary physiological changes. Each of the PGRs has the potentiality to alter almost all aspects of plant growth and development as both promoter as well as retardant, when used in appropriate concentration.

In the present experiment the plants were exposed to different PGRs such as GA_3, IAA, IBA, BAP, Kn, SA, Tria, MJ and CCC, in order to improve herb yield and to enrich their content with functional oil constituents, beneficial for human health.

5.1 Effect of PGRs on growth parameters

The improvement in growth characters of peppermint resulted from the foliar application of SA, Kn, Tria and MJ over the control (Table 4) is understandable. The SA mediated enhanced growth of the plants can be associated with the regulatory effects of SA on cell growth and division. It has been suggested that the growth promoting effects of SA could be related to change in the hormonal status (Shakirova *et al.*, 2003, Abreu and Munne-Bosch, 2009) or by improvement of photosynthesis, transpiration and stomatal conductance (Stevens *et al.*, 2006). Application of Kn, Tria and MJ also exhibited a vital influence on biomass accumulation. The stimulatory effect of Kn on fresh and dry weight accumulation in plants can be attributed to its role in stimulating leaf area expansion, chlorophyll accumulation and dry matter production in plants. A positive role of Kn was also reported by Davies (1995) and Pospisilova *et al.* (2000). The stimulatory effects of Tria may be due to the rapid translocation of Tria throughout the plant, causing a cascade of metabolic events and resulting in significant increase in growth and dry matter. The results on the ameliorative effect of Tria broadly confirms the findings of Srivastava and Sharma (1990), Misra and Srivastava (1991), Shukla *et al.* (1992), Muthuchelian *et al.* (2003), Giridhar *et al.* (2005) and Chaudhary *et al.* (2006).

5.2 Effect of PGRs on physio-biochemical parameters

PGRs are also known to influence apart from morphological characters, different physiological and biochemical parameters. The influence of the various growth promoting and retarding substances in comparison with the control on chlorophyll content, carotenoid content, NR and CA activities, leaf N, P and K contents of peppermint is discussed hereunder.

The perusal of data (Table 5) reveals that there was a positive effect of SA, Tria and MJ on total chlorophyll content and carotenoid content of leaves. The data on the positive effect of SA were in agreement with those of Aftab *et al.* (2010) who suggested that foliar spray with SA increased total chlorophyll and carotenoid contents in *Artemisia* plants. This was possibly because of the role of the PGR in delaying the senescence and abscission, functioning of stomatal regulation and transpiration, respiratory pathway as well as production of chlorophyll as reported by various workers including Menon and Srivastava (1984), Fariduddin *et al.* (2003),

Popova *et al.* (1997), Yusuf *et al.* (2008) and Abreu and Munne-Bosch (2009). The improvement in chlorophyll content, growth and dry weight of plants treated with Tria may be attributed to an improvement in photosynthesis and an enhanced accumulation of photosynthates. A correlation between the higher transcription of the rbcS gene and the improved photosynthetic activity in Tria-treated plants was reported by Chen *et al.* (2003). Moreover, GA_3 treatment showed a significant decrease in chlorophyll and carotenoid levels. Similar results were also obtained by Misra *et al.* (2009) on *Catharanthus roseus* and Garg and Kumar (2012) on *Euphorbia lathyris*. There was an increase in the contents of chlorophyll and carotenoid due to application of the growth retardant. An increase in chlorophyll content with the application of growth retardant CCC was also reported earlier by Farooqi *et al.* (2003) and Shalaby *et al.* (2007).

The enzyme NR catalyses the reduction of nitrate to nitrite and is a rate limiting step in the nitrogen metabolism (Beevers and Hageman, 1969). It has been observed that the activity of NR increased significantly with the application of the PGRs, with Tria giving the maximum NR activity (Table 6). Similarly, Sivakumar *et al.* (2002) also reported an increase in NR activity in pearl millet due to foliar application of Tria. CA catalyses the reversible hydration of CO_2, thereby, making available the ribulose-1. 5-bisphosphate carboxylase/oxygenase (RuBisCO) in the chloroplast stroma. The data (Table 6) showed that SA increased CA activity. A probable reason for the enhancement of the activity might be the de novo synthesis of CA, which involves translation/transcription of the associated genes (Okabe *et al.*, 1980). Induction of carboxylation efficiency by SA was also reported earlier by Fariduddin *et al.* (2003) and Hayat *et al.* (2010).

Considering the effect of PGRs on leaf nutrient contents, SA increased leaf N content immensely whereas Kn, leaf P and leaf K compared with the control (Table 7). Other PGRs such as Tria, GA_3, BAP and MJ have also significant effect on the leaf N, P and K contents. The enhancement in leaf N, P and K contents due to the PGRs might be related to the enhanced permeability of membranes resulting from the action of PGRs (Al-Wakeel *et al.*, 1995; Naeem *et al.*, 2010, 2011). An increase in membrane permeability would facilitate absorption and utilization of mineral nutrients and also transport of assimilate (Ansari, 1996; Khan *et al.*, 1998). This would also contribute towards enhancing the capacity of the treated plants for biomass production as

reflected in the observed increase in fresh and dry weights of plants (Table 4). These findings broadly corroborate the observations of Chaudhary *et al.* (2006) and Naeem *et al.* (2009).

5.3 Effect of PGRs on yield and quality characteristics

The improvement in essential oil content and essential oil yield due to application of SA, Tria, CCC and MJ over control treatments in peppermint (Table 8) is not far to seek. The positive effect of SA on essential oil content and essential oil yield may be ascribed to the improvement in overall plant growth and metabolism by SA. It seems that SA might enhance the intrinsic genetic potential of the mint plants to produce additional herbage yield with improved quantity of essential oil. Thus, SA mediated enhanced plant growth, photosynthesis and the overall plant metabolism might have accounted for oil accumulation in the present study. In fact, a photosynthetic model in relation to essential oil production in *Mentha piperita* L. was proposed by Burbott and Loomis (1967) which was later modified by Clark and Menary (1980). The model showed that the balance between production and utilization of photosynthates was an important determinant of oil accumulation and major components of the oil. As per the model, the rates of photosynthesis as well as the factors affecting photosynthesis were suggested to be the determining factors regarding oil accumulation (Srivastava and Sharma, 1991).The enhanced essential oil yield in SA treated plants might also be ascribed to the increased uptake of leaf nutrients (N, P and K) that could have been subsequently used to enhance the formation of photosynthates and other metabolites with regard to oil formation. Our results in this regard corroborate the findings of Aftab *et al.* (2010), Rowshan *et al.* (2010), Rahimi *et al.* (2013) on *Cumium cynium* and Gharib *et al.* (2007) on *Ocimum basilicum* and *Majoran hortensis*. The improving effect of Tria on content and yield of peppermint oil over the control (Table 8) may be ascribed to the assumption that Tria might enhance the intrinsic genetic potential of the mint plants to produce additional quantity of essential oil. The enhanced essential oil yield in Tria-treated leaves might also be ascribed to the increased uptake of leaf-nutrients (N, P and K) that could have been subsequently used to enhance the formation of photosynthates and other metabolites with regard to oil formation.

The enhancement of the oil content in CCC treated plants over the control (Table 8) may be traced to the well-known fact that essential oil is derived from mevalonic acid via the isoprene pathway in a manner similar to that for other terpenes. Growth

retardants such as phosphon D and CCC which have been shown to increase terpene formation resulting in increased essential oil content of peppermint and sage (El-Keltawi and Croteau, 1987). Similar results were also reported by Farooqi (1988) on *Mentha arvensis* and Saffari *et al.* (2004) on *Rosa damascena.* MJ treated plants have also significantly higher essential oil content and yield. It was reported that jasmonate can act as powerful inducing signals for biosynthesis of secondary metabolites. The increased amount of essential oil due to treatment of MJ was believed to be due to an increased level of H_2O_2 production followed by phenylalanine ammonia lyase activity and there are many evidence that prove that H_2O_2 act as a diffusible signal to activate defense genes and the biosynthesis of plant secondary metabolites. (Mehdy, 1994; Mithofer *et al.*, 1997; Qian *et al.*, 2004; Choi *et al.,* 2005; Zheliazkov *et al.*, 2010). Li *et al.* (2007) revealed many of transcripts displaying high similarities to the known enzymes and peptide linked to the formation of secondary metabolites in sweet basil that it was affected by MJ.

The decrease oil content and oil yield in GA_3-treated plants (Table 8) may be traced to the fact that because gibberellins are diterpenes and they regulate their own metabolism, deviating or inhibiting the transcription of genes which codify enzymes for gibberellin synthesis and degradation pathways (feedback and feed-forward regulations, respectively). Thus, the level of active gibberellins is kept with slight variation, as long as precursors are available and biosynthesis and degradation enzymes are functional. Therefore, gibberellin application results in a lower expression of GA_{20}-oxidase and GA_3-oxidase biosynthetic genes and a higher transcription of GA_2-oxidase degradation gene. Consequently, GA_3 application might have caused endogenous gibberellin degradation, which resulted in low essential oil content and yield.

The increase in menthol content and acidic oil due to SA and GA_3 application respectively over the control (Table 9 and 11) might be possible due to higher synthesis of menthol and acetate respectively in SA treated plants. These results broadly confirm the findings of Srivastava and Sharma (1991), Singh *et al.* (1999) and Sangwan *et al.* (2001) on *Mentha.*

5.4 CONCLUSION

The evaluation of the obtained results in the present study allows the present author to conclude that application of the PGRs enhanced the growth physiological, biochemical and yield and quality characteristics of plants.

The spray of SA (10^{-6} M) resulted in the highest oil content and oil yield.

Moreover, SA treatment increased the menthol content, BAP spray increased the menthone content, whereas GA_3 increased menthyl acetate content maximally.

Table 12. Correlation coefficient (r) values for different pairs of characteristics of peppermint

Characteristics	Fresh weight per plant	Dry weight per plant	Chlorophyll content	Carotenoid content	Nitrate reductase activity	Carbonic anhydrase activity	Nitrogen content	Phosphorus content	Potassium content
Fresh weight per plant	-	0.986	0.549	0.780	0.510	0.927	0.875	0.226	0.469
Dry weight per plant	-	-	0.642	0.810	0.517	0.940	0.920	0.189	0.373
Chlorophyll content	-	-	-	0.866	0.388	0.624	0.699	-0.317	-0.118
Carotenoid content	-	-	-	-	0.301	0.698	0.743	-0.168	0.085
Nitrate reductase activity	-	-	-	-	-	0.697	0.639	0.245	0.606
Carbonic anhydrase activity	-	-	-	-	-	-	0.905	0.149	0.421
Nitrogen content	-	-	-	-	-	-	-	0.232	0.250
Phosphorus content	-	-	-	-	-	-	-	-	0.535
Potassium content	-	-	-	-	-	-	-	-	-

	r value
Significant at 5%	0.765
Significant at 1%	0.623

Contd....

Table 12 (Contd.) Correlation coefficient (r) values for different pairs of characteristics of peppermint

Characteristics	Essential oil content in leaves	Essential oil yield per plant	Menthol content in the oil	Menthol yield per plant	Menthone content in the oil	Menthone yield per plant	Menthyl acetate content in the oil	Menthyl acetate yield per plant
Fresh weight per plant	0.294	0.626	0.487	0.684	-0.193	0.533	-0.117	0.580
Dry weight per plant	0.367	0.682	0.408	0.728	-0.179	0.548	-0.130	0.641
Chlorophyll content	0.821	0.884	-0.022	0.844	-0.082	0.686	-0.289	0.829
Carotenoid content	0.736	0.883	0.103	0.861	-0.042	0.740	-0.341	0.807
Nitrate reductase activity	0.156	0.348	0.492	0.395	-0.336	0.543	0.426	0.405
Carbonic anhydrase activity	0.316	0.634	0.484	0.691	-0.320	0.607	-0.070	0.590
Nitrogen content	0.457	0.711	0.304	0.725	-0.231	0.591	0.126	0.749
Phosphorus content	-0.622	-0.459	0.070	-0.455	0.487	-0.120	0.306	-0.360
Potassium content	-0.381	-0.117	0.725	-0.012	0.004	0.084	0.224	-0.120

	r value
Significant at 5%	0.765
Significant at 1%	0.623

Contd....

Table 12 (Contd.) Correlation coefficient (r) values for different pairs of characteristics of peppermint in Experiment

Characteristics	Essential oil content in leaves	Essential oil yield per plant	Menthol content in the oil	Menthol yield per plant	Menthone content in the oil	Menthone yield per plant	Menthyl acetate content in the oil	Menthyl acetate yield per plant
Essential oil content in leaves	-	0.922	0.143	0.860	-0.360	0.590	-0.130	0.906
Essential oil yield per plant	-	-	-0.141	0.985	-0.421	0.664	-0.131	0.968
Menthol content in the oil	-	-	-	0.299	-0.485	-0.032	0.299	0.144
Menthol yield per plant	-	-	-	-	-0.495	0.613	-0.105	0.946
Menthone content in the oil	-	-	-	-	-	-0.073	-0.327	-0.449
Menthone yield per plant	-	-	-	-	-	-	-0.113	0.619
Menthyl acetate content in the oil	-	-	-	-	-	-	-	0.102

	r value
Significant at 5%	0.765
Significant at 1%	0.623

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APPENDIX

APPENDIX

1. PREPARATION OF STOCK SOLUTIONS OF PGRs (10^{-3} M concentration)

(i) IAA

17.5 mg of IAA was dissolved in10 mL 1N NaOH and the final volume was made 100 mL using DDW.

(ii) IBA

17.5 mg of IBA was dissolved in10 mL 1N NaOH and the final volume was made 100 mL using DDW.

(iii) GA_3

34.63 mg GA_3 was dissolved in 10 mL ethyl alcohol and the final volume was made 100 mL using DDW.

(iv) Kn

21.5 mg of Kin was dissolved in 10 mL 1N NaOH and the final volume was made 100 mL using DDW.

(v) BAP

22.5 mg of BAP was dissolved in 10 mL 1N NaOH and the final volume was made 100 mL using DDW.

(vi) Tria (0.05% solution)

87.6 mL of Tria was dissolved in 100 mL of DDW.

(vii) SA Stock solution

13.8 mg of SA was dissolved in 100 mL of DDW.

(viii) MJ

0.026 mL of MJ was dissolved in10 mL ethanol and the final volume was made 100 mL using DDW.

(ix) CCC (51.60 % solution)

0.030 mL of CCC was dissolved in10 mL ethanol and the final volume was made 100 mL using DDW.

2. PREPARATION FOR VARIOUS REAGENTS

(i) Reagents for chlorophyll and carotenoid contents

80% acetone

80 mL of acetone was mixed in 20 mL of DDW.

(ii) Reagents for nitrate reductase activity

Isopropanol (5%)

5 mL isopropanol was mixed in 95 mL DDW.

N-1-nephthyl-ethylenediamine-dihydrochloride solution (0.02% N): (NED-HCl)

20 mg NED-HCl was dissolved in sufficient DDW and final volume was made up to 100 mL with DDW.

Phosphate buffer (0.1M) of pH 7.4

27.2 g KH_2PO_4 and 45.63 g $K_2HPO_4.7H_2O$ were dissolved separately in sufficient DDW and final volume was made up to 1000 mL with DDW. The above solutions of KH_2PO_4 and $K_2HPO_4.7H_2O$ were mixed in the ratio 16:84.

Potassium nitrate solution (0.2 M)

20.02 g potassium nitrate was dissolved in sufficient DDW and final volume was made up to 1000 mL with DDW.

Sulphanilamide solution (1%)

1 g sulphanilamide was dissolved in 100 mL 3N HCl which was prepared by mixing 26.53 mL HCl into 73.47 DDW.

(iii) Reagents for carbonic anhydrase activity

Cystein hydrochloride solution (0.2 M)

48 g of cystein hydrochloride was dissolved in sufficient volume of DDW and the final volume was made up to 1000 mL with DDW.

Phosphate buffer (0.2 M) of pH 6.8

5.365 g of Na_2HPO_4 and 2.78 g of $NaH_2PO_4.H_2O$ were dissolved separately in sufficient DDW and final volume of each was made up to 100 mL using DDW. To get pH 6.8, the above solution of Na_2HPO_4 and $NaH_2PO_4.2H_2O$ were mixed in the ratio of 49:51.

Sodium bicarbonate solution (0.2 M) in 0.02 M NaOH

16.8 g sodium bicarbonate was dissolved in NaOH solution (0.8 g NaOH/L) and final volume was maintained up to 1000 mL with NaOH solution.

Bromothymol blue indicator in ethanol (0.002%)

0.002 g of bromothymol blue was dissolved in 10 mL ethyl alcohol and final volume was made up to 100 mL, by using DDW.

Hydrochloric acid (0.05 N)

0.44 mL pure hydrochloric acid was mixed using 99.5 mL DDW.

Methyl red

Methyl red at 100 mg was dissolved in sufficient ethanol and final volume was made 100 mL using ethanol.

(iv) Reagents for nitrogen, phosphorus and potassium contents

Reagents for nitrogen

Sodium hydroxide solution (2.5 N)

100 g NaOH dissolved in sufficient DDW and final volume was made up to 1000 mL with DDW.

Sodium silicate solution (10%)

10 g sodium silicate dissolved in sufficient DDW and final volume was made up to 100 mL with DDW.

Reagents for phosphorus

Molybdic acid reagent (2.5%)

6.25 g of ammonium molybdate was dissolved in 175 mL 10 N H_2SO_4 which was made by mixing 27.2 mL H_2SO_4 with 72.8 mL DDW

1-Amino-2-naphthol-4-sulphonic acid

0.5 g 1-amino-2-naphthol-4-sulphonic acid was dissolved in 195 mL of 15% sodium bisulphite solution to which 5 mL of 20% sodium sulphite solution was added. The solution was kept in amber colored bottle.

Sodium bisulphite solution (15%) was made by dissolving 15 g sodium bisulphite in sufficient amount of DDW and the final volume was maintained up to 100 mL using DDW.

Sodium sulphite solution (20%) was made by dissolving 20 g sodium sulphite was dissolved in sufficient amount of DDW and the final volume was maintained up to 100 mL using DDW.